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# BRILLIANT GREEN BROTH AS A SPECIFIC ENRICHMENT MEDIUM FOR THE PARATYPHOID-ENTERITIDIS GROUP OF BACTERIA.\*

JOHN C. TORREY.

(From the Department of Experimental Pathology, Loomis Laboratory, Cornell University Medical College, New York City.)

Altho malachite green as an ingredient of solid medium was introduced by Loeffler primarily as an aid in the isolation of the typhoid bacillus, subsequent investigations have demonstrated the greater selective action of this dye for bacilli of the paratyphoid-enteritidis type. Loeffler<sup>1</sup> in 1907 described a nutrose-peptone-lactose-malachite green agar medium which exerts a marked inhibitory action on the development of bacilli of the colon type, but is favorable for the growth of paratyphoid bacilli. This medium, apparently, has given good results in Germany, but as it is rather complicated and troublesome to prepare it does not seem to have been widely adopted. In the following pages is described a very simple and inexpensive fluid medium which may be prepared readily in almost any laboratory and yet which, according to our experiments, exhibits a higher degree of selective availability for growth and isolation of the paratyphoid-enteritidis group than does Loeffler's malachite green solid medium.

Malachite green and brilliant green are both basic dyes and, according to Arthur G. Green,<sup>2</sup> are closely related. In 1908 Conrad<sup>3</sup> suggested the use of brilliant green instead of malachite green as one of the ingredients of a solid medium for the typhoid bacillus, primarily for the reason that brilliant green does not interfere with typhoid agglutination tests. According to the writer's experience neither of these dyes affects the agglutinability of bacilli of the paratyphoid group, and so far as that test is concerned it is a

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<sup>1</sup> *Deutsch. med. Wchnschr.*, 1907, 33, p. 1581.

<sup>2</sup> *Organic Colouring Matters*, London, 1908. The nature of these two dyes is described under the heading "Triphenylmethane and Diphenylnaphthylmethane Colouring Matters," p. 180.

<sup>3</sup> *Centralbl. f. Bakteriöl., Referate*, 1908, 42, Beiheft 1, p. 47.

matter of indifference which dye is used, but in the inhibition of the growth of the colon group and other typically fecal bacteria, brilliant green<sup>1</sup> is more potent than is malachite green and yet it is rather more favorable for the multiplication of bacilli of the paratyphoid-enteritidis group. In the experiment detailed in Table 1, the specified amounts of one per cent aqueous solutions of the dyes (Grübler's) mentioned were added to 10 c.c. of glucose

TABLE 1.  
COMPARATIVE TEST OF MALACHITE GREEN (HÖCHST 120) AND BRILLIANT GREEN.

Tube	Dye	Amount of 1 Per Cent Aqueous Solution of Dye Added to 10 c.c. Glucose Broth. c.c.	Cultures Seeded	Number of Bacilli Seeded per 0.5 c.c.	Bacilli per 0.1 c.c. after 48 Hours' Incubation
1.....	Brilliant green	0.15	B. coli mixture	7,000	0
2.....	Malachite	0.15	" " "	"	∞
3.....	"	0.3	" " "	"	23,000
4.....	"	0.6	" " "	"	0
5.....	"	1.0	" " "	"	0
6.....	Brilliant	0.15	B. paratyphosus B	6	∞
7.....	"	0.3	" " "	"	∞
8.....	"	0.6	" " "	"	∞
9.....	Malachite	0.15	" " "	"	∞
10.....	"	0.3	" " "	"	∞
11.....	"	0.6	" " "	"	0
12.....	"	1.0	" " "	"	∞
13.....	"	2.0	" " "	"	0
14.....	Brilliant	0.15	B. enteritidis	6	∞
15.....	"	0.3	" " "	"	∞
16.....	"	0.6	" " "	"	∞
17.....	Malachite	0.15	" " "	"	∞
18.....	"	0.3	" " "	"	∞
19.....	"	0.6	" " "	"	∞
20.....	"	1.0	" " "	"	100,000
21.....	"	2.0	" " "	"	200

broth, neutral to phenolphthalein. A comparison of Tubes 1-4 shows that 0.15 c.c. of this brilliant green solution (a final dilution of 1-6,600) killed a fairly heavy seeding of a mixture of bacilli of the colon group consisting of *B. coli communis*, *B. coli communior*, *B. acidi lactici*, and *B. lactis aërogenes*, but with malachite green (Höchst 120) an addition of 0.3 to 0.6 c.c. of a one per cent aqueous solution was required to effect the same result. Further, 0.6 c.c. of malachite green which was bactericidal for the colon mixture (Tube 4) also destroyed *B. paratyphosus* B (Tube 11), whereas the

<sup>1</sup> As far as I am aware brilliant green has not been suggested heretofore as an ingredient of an enrichment medium for the paratyphoid group. In 1908 Peabody and Pratt (*Boston Med. and Surg. Jour.*, 1908, 158, p. 213) described a malachite green enrichment-broth medium for the typhoid bacillus, but there are few data available in regard to its efficiency.

same amount of brilliant green checked in no degree the multiplication of this paratyphoid bacillus nor that of *B. enteritidis*. The irregular results in Tubes 11, 12, and 13 were probably due to the fact that the malachite green when added to the broth menstruum in the specified amounts underwent more or less precipitation. These experiments as a whole indicate the superiority of brilliant green over malachite green as an ingredient in an enrichment medium for the paratyphoid-enteritidis group.

After a series of tests of different degrees of acidity of broth combined with varying strengths of brilliant green, the following combination was found to give the optimum differential results. Meat-peptone-broth, prepared according to the usual methods, is titrated to the neutral point for phenolphthalein and one per cent glucose is added. The medium is tubed in exactly 10 c.c. amounts and sterilized in the Arnold. The final reaction must be close to neutral. A one per cent solution of brilliant green (Grüb-ler's) in distilled water is next prepared. This does not require sterilization. Just before the medium is to be used, 0.15 c.c. of this brilliant green solution is added to each tube. This constitutes a final dilution of approximately 1-6,600, which, as has been stated, is a strength sufficient to inhibit or destroy the dominant fecal bacteria and yet exercises no repressive action on members of the paratyphoid-enteritidis group. With the given amount of brilliant green a slight increase in the acidity of the broth causes a marked increase in the bactericidal properties of the medium. At 0.5 acid to phenolphthalein only certain strains of the paratyphoid and enteritidis bacillus would grow, while at 1.0 acid none of a number of strains of this group grew.

An enrichment medium, made according to the methods described above, has been tested with a number of representatives of the paratyphoid-enteritidis group and with other bacteria which occur constantly or occasionally in the intestinal tract. These cultures have been received from a number of different sources and have been adequately identified. All of the cultures<sup>†</sup> placed in the

<sup>†</sup> For cultures numbered 5, 9, 10, 15, 16, 17, 30, 32, and 33 in Table 2, I gratefully acknowledge my indebtedness to Professor C.-E. A. Winslow, Department of Public Health, American Museum of Natural History; and for cultures 3, 4, 6, and 7, to Dr. Krumwiede, Health Department Research Laboratory New York City.

left-hand column belong definitely in the paratyphoid-enteritidis group, except No. 18 which is a bacillus isolated from dog feces and similar to *B. enteritidis* except that it acts slowly on lactose and is not pathogenic when fed to mice. All of these cultures belonging in the "intermediate group" grew readily in this brilliant green broth except Schottmüller No. 8. This strain had apparently become atypical through conditions of cultivation, because Schottmüller No. 7, which is undoubtedly its original parent strain, multiplied actively in this medium. Of other bacteria, selected as controls, the only species which grew readily in this medium was *B. pyocyaneus*. For other cultures the dye proved either inhibitory or rapidly bactericidal. The pyogenic cocci, *B. dysenteriae*, *B. alkaligenes*, Morgan's bacillus No. 1, and certain strains of *B. coli*, were killed within a few minutes in this medium. This strength of brilliant green also proved toxic for the typhoid bacillus. Certain strains belonging in the colon group, especially *B. coli communior* and *B. acidi lactici*, might exhibit a moderate increase after two to four hours' incubation, but after 24 hours were found to have practically died out. The glucose was added to the medium with the idea that organisms only moderately susceptible to the strength of dye, at the neutral point, by splitting this sugar might so increase the acidity of the medium as to cause their own destruction, for a slight increase in acidity greatly enhances the toxicity of brilliant green. Of the members of the colon group *B. lactis aërogenes* shows the greatest degree of tolerance for this dye, but our experiments have shown that *B. enteritidis* is able to overgrow completely and eliminate this bacillus even when the latter is present originally in much smaller numbers. It may be observed that the seeding of the control cultures was made in general much heavier than that of the paratyphoid-enteritidis strains.

The selective action of brilliant green on groups of bacteria has not been studied as extensively as has that of gentian violet by Churchman.<sup>1</sup> It would seem, however, that brilliant green exerts the finer degree of selective action. Relatively few bacteria have been found with a tolerance for this stain and they were almost exclusively confined to the paratyphoid-enteritidis group. It is

<sup>1</sup> *Jour. Exper. Med.*, 1912, 16, pp. 221 and 822.

possible that other members of the large mucosus capsulatus group may be able to multiply readily in this medium, but as the matter was not pertinent to the purpose of this investigation no tests were made.

TABLE 2.  
SHOWING THE SELECTIVE ACTION OF BRILLIANT GREEN GLUCOSE BROTH FOR BACILLI OF THE  
PARATYPHOID-ENTERITIDIS GROUP.

Cultures	Plated at Once to Determine Number of Bacteria Seeded. Result per 0.1 c.c.	Plated after 24 Hours' Incubation at 37° C. Result per 0.1 c.c.	Cultures	Plated at Once. Bacteria in 0.1 c.c.	Plated after 24 Hours' Incubation
1. <i>B. paratyphosus</i> A (7) . .	150	∞	19. <i>B. coli</i> communis . . .	1,690	0
2. " " " (116) . .	975	∞	20. " " " . . .	8,320	520
3. " " " (Y) . .	1,430	∞	21. " " communior . . .	7,800	1
4. " (Seeman) . . . . .	250	∞	22. " " " . . .	8,800	100
5. " " B (22) . .	485	∞	23. <i>B. acidi lactici</i> . . . .	9,900	14
6. " " " (Y) . .	1,560	∞	24. " " " . . . . .	11,500	80
7. " (Schottmüller) . . . .	300	∞	25. " " " . . . . .	2,145	0
8. <i>B. paratyphosus</i> B (Schottmüller) . . . .	2,275	3,900	26. <i>B. lactis aërogenes</i> . .	15,500	40,000
9. <i>B. enteritidis</i> (132) . . . .	2,340	∞	27. <i>B. typhosus</i> (79) . . . .	1,560	1
10. " " (18) . . . . .	910	∞	28. <i>B. dysenteriae</i> (Kruze) . .	13,000	20,000
11. " " (Dog 69) . . . . .	1,300	∞	29. <i>B. dysenteriae</i> (Flexner) . .	8,500	10
12. " " (Dog 59) . . . . .	2,470	∞	30. Morgan's bacillus No. 1 . .	6,000	0
13. " " (Dog 40) . . . . .	975	∞	31. <i>B. alkaligenes</i> (439) . .	19,000	35
14. " " (Rat) . . . . .	3,250	∞	32. <i>B. proteus vulgaris</i> 1 . .	16,000	0
15. <i>B. suispestifer</i> . . . . .	1,820	∞	33. " " " 2 . . . . .	3,000	0
16. <i>B. typhi murium</i> . . . . .	1,300	∞	34. <i>B. pyocyaneus</i> . . . . .	6,000	70
17. <i>B. danysz</i> . . . . .	2,200	∞	35. <i>Aur. aureus</i> . . . . .	4,000	900
18. <i>Bacillus</i> No. 125 . . . . .	910	∞	36. <i>Alb. pyogenes</i> . . . . .	9,500	0
			37. <i>Str. pyogenes</i> . . . . .	2,000	0

In determining the proper strength of brilliant green for this medium, an attempt was made to select that dosage which would not inhibit in any degree the growth of the paratyphoid-enteritidis group, but yet would exert a strong repressive action on the commonly occurring fecal bacteria. The experiment detailed in Table 3 shows that the strength of the dye adopted did not inhibit the development of a typical culture of *B. enteritidis* nor of *B. paratyphosus* B, even when the total seeding amounted to only 4 or 5 bacteria, growth occurring as readily in the brilliant green broth as in the same broth without the dye. For two strains of *B. paratyphosus* A this also held true, but one strain, 116, proved more sensitive to this stain as there occurred no growth even when the total seeding amounted to over 600 bacilli. With a heavier seeding, however, this strain will also grow readily (Table 2, No. 2).

As a rule in an infected stool the paratyphoid bacilli are greatly outnumbered by the normal fecal flora and it is evident that a selective fluid medium to be of value must be so constituted that bacilli of the paratyphoid group may overgrow relatively large

TABLE 3.  
SEEDING OF SMALL NUMBERS OF *B. paratyphosus*, TYPES A AND B, INTO BRILLIANT GREEN GLUCOSE BROTH AND INTO GLUCOSE BROTH TO DETERMINE THE COMPARATIVE TENDENCY TO GROWTH.

Dilution Tube	Organism Seeded	Total Number of Bacilli Seeded	Brilliant Green Glucose Broth Tubes after 24 Hours' Incubation	Glucose Broth Tubes after 24 Hours' Incubation
5.....	<i>B. paratyphosus</i> , Type A	84	Growth, dark green, gas.	Growth
6.....	<i>B. paratyphosus</i> , Type A	3	No growth	"
7.....	<i>B. paratyphosus</i> , Type A	0	" "	No growth
5.....	<i>B. paratyphosus</i> Type A (7)	230	Growth	Growth
6.....	<i>B. paratyphosus</i> Type A (7)	11	"	"
7.....	<i>B. paratyphosus</i> Type A (7)	2	"	"
4.....	<i>B. paratyphosus</i> Type A (116)	620	No growth	Growth
5.....	<i>B. paratyphosus</i> Type A (116)	29	" "	"
6.....	<i>B. paratyphosus</i> Type A (116)	6	" "	No growth
5.....	<i>B. enteritidis</i> No. 18	80	Growth	Growth
6.....	" " " "	4	"	"
7.....	" " " "	0	No growth	No growth
5.....	<i>B. paratyphosus</i> (Schottmüller)	90	Growth	Growth
6.....	<i>B. paratyphosus</i> (Schottmüller)	5	"	"
7.....	<i>B. paratyphosus</i> (Schottmüller)	0	No growth	No growth

numbers of these fecal bacteria. The following experiment (Table 4) was designed to determine to what extent this medium is effective as regards the colon group. The colon seeding consisted of a mixture of cultures of *B. coli communis*, *B. coli communior*, *B. lactis aërogenes*, and *B. acidi lactici*. It was found that all of the three representatives of the paratyphoid-enteritidis group when present in the ratio of one paratyphoid to 250 or 300 colon bacilli readily overgrew them to the extent that after 24 hours' incubation pure cultures were present in the tubes. In a ratio of one paratyphoid bacillus up to 180,000 colon bacilli this in large measure still held true, whereas when the colon seeding amounted to 1,800,000 bacilli to one *B. enteritidis*, the latter apparently was unable to multiply.

In view of the fact that malachite green agar is generally recognized as a favorable medium for the isolation of bacilli of the paratyphoid group, any substitute offered may reasonably be expected to equal or surpass its efficiency as a selective medium for the isolation of the paratyphoid group, aside from any other advantages which such a substitute may offer in the way of greater simplicity in preparation. Accordingly comparative tests have been undertaken in which the same samples of infected feces were cultivated on Loeffler's malachite green-nutrose-lactose agar, especially devised by the originator for paratyphoid work, and in the brilliant green glucose broth.

TABLE 4.  
COMBINED SEEDING OF *B. coli* MIXTURE AND *B. enteritidis* OR *B. paratyphosus* IN BRILLIANT GREEN GLUCOSE BROTH.

TOTAL SEEDING		TUBES INCUBATED 24 HOURS AND PLATED ON ENDO MEDIUM
<i>B. coli</i> Mixture	<i>B. enteritidis</i> or <i>B. paratyphosus</i>	
920,000	<i>B. enteritidis</i> 35,000	Pure culture, <i>B. enteritidis</i>
"	" 6,000	" " "
"	" paratyphosus B 8,000	" " <i>B. paratyphosus</i>
"	" 3,200	" " " "
"	" (Seeman), 17,500	" " " "
"	" 6,000	" " " "
28,000,000	" enteritidis 3,900	Nearly pure culture, <i>B. enteritidis</i>
"	" 360	" " " "
"	" 160	Few <i>B. coli</i> , no " "
"	" 120	Nearly pure culture " "
285,000,000	" 3,900	<i>B. coli</i> reduced, no " "
"	" 360	" " " "
Control 920,000	None	No growth
" 28,000,000	"	<i>B. coli</i> , 160,000 per 0.1 c.c.
" 285,000,000	"	" 1,000,000 " " "

For the purpose of these comparative tests dogs were fed with boiled milk seeded with 24-hour agar or broth cultures of various strains of the paratyphoid-enteritidis group. It may be noted in passing that the feeding seemed to cause no definite ill effect to the subjects. Generally a transitory diarrhea occurred lasting a day or two, but no other indication of toxic action. This result agrees with the well known natural immunity of dogs to this group of bacteria.

At daily intervals the stools of these subjects were cultivated on the Loeffler malachite green agar and also in the brilliant green glucose broth. Two or three plates of the Loeffler medium were seeded from each specimen of feces. In addition to seeding from

thick emulsions of the feces, a plate was generally streaked with a loop from the undiluted stool. Success attended the second mode of seeding more often than the first. In cultivating the feces with the brilliant green glucose broth the following method was found to give the best results; in fact any deviation from it was likely to be attended with failure.

*Method for use of medium in cultivating feces.*—To six tubes of 10 c.c. each of glucose broth, neutral to phenolphthalein, 0.15 c.c. of a one per cent aqueous solution of brilliant green is added. It is advisable to add the dye to each tube of broth just before it is to be used as after the lapse of a day or two a slight amount of precipitation will have occurred with a consequent weakening of the medium. In one of these tubes of brilliant green broth from one-fourth to one-half a gram of feces is thoroughly emulsified. This is allowed to settle for about 10 minutes, then 1 c.c. of this emulsion is seeded into one tube of brilliant green broth, 0.5 c.c. into each of two tubes, and three drops into each of two tubes. If the paratyphoid or enteritidis bacilli are fairly numerous in the feces, pure or nearly pure cultures will develop in all of these tubes. If, however, they are present in very small numbers, the specific growth occurs rather more often in the lightly than in the heavily seeded tubes. If *B. lactis aërogenes* is plentiful in the feces it may multiply, but apparently may be overgrown by *B. paratyphosus* B or *B. enteritidis*, even tho at the start the latter are present in the great minority.

The tubes, with the exception of the primary emulsion which is discarded, are incubated for 24 hours at 37° C. Growth is generally indicated by gas formation and a darkening in the shade of green. Streak cultures are then made on Endo plates to determine the type of growth and typical colonies are isolated and subjected to the cultural and agglutination tests necessary for identification. As paratyphoid or enteritidis bacilli when present at all in the incubated tubes are generally in pure or nearly pure culture, early information may be gained by adding a drop of strong specific agglutinating serum to the tube. This test obviously cannot take the place of isolation and proper identification.

In Table 5 are assembled the data in regard to the practical comparative tests of the efficiency of these two media. In the total 18 experiments of this character the bacillus fed to the dogs was recovered, using the Loeffler malachite green plates, in nine instances or 50 per cent, but with the brilliant green broth in 14 instances or 77.7 per cent. Furthermore in five experiments the bacillus fed was isolated with the enrichment medium where it could not be recovered from the Loeffler plates, whereas in no instance was the bacillus recovered with the plate medium where it was not found growing in the brilliant green broth. Aside from its greater efficiency the simplicity of this brilliant green enrichment medium is

much in its favor. In any bacteriological laboratory it could be prepared within an hour or two for an emergency.

TABLE 5.

COMPARATIVE RESULTS IN CULTIVATING THE FECES OF DOGS FED WITH *B. paratyphosus* OR WITH *B. enteritidis* ON LOEFFLER'S MALACHITE GREEN AGAR AND IN BRILLIANT GREEN GLUCOSE BROTH.

Dog	Dose	Examination of Feces after Feeding (Days)	Endo Plates	Loeffler's Malachite Green Plates	Brilliant Green Broth
138.....	<i>B. enteritidis</i> ; 3 broth cultures	4	None made	Negative	Positive; pure culture <i>B. enteritidis</i> in 2 tubes.
139.....	<i>B. enteritidis</i> ; 3 agar cultures	2	Negative	Positive; 4 colonies on 2 plates	Positive; pure culture in all 4 tubes.
139.....	<i>B. enteritidis</i> ; 3 agar cultures	3	"	Positive; 2 colonies	Positive; pure culture <i>B. enteritidis</i> in all 4 tubes.
139.....	<i>B. enteritidis</i> ; 3 agar cultures	4	"	Positive; 1 colony	Positive; pure culture in 1 tube, mixed in others.
140.....	<i>B. enteritidis</i> ; 1 broth culture	1	Negative	Positive; many colonies	Positive; pure culture in 2 tubes.
140.....	<i>B. enteritidis</i> ; 1 broth culture	2	"	Positive; fairly numerous	Positive; almost pure in 4 tubes.
140.....	<i>B. enteritidis</i> ; 1 broth culture	3	"	Positive; 2 colonies	Positive; pure culture in 1 tube.
140.....	<i>B. enteritidis</i> ; 1 broth culture	4	"	Negative	Positive; pure culture in 1 tube.
142.....	<i>B. enteritidis</i> ; 3 agar cultures	3	"	Positive; 2 colonies	Positive; pure in 1 tube.
142.....	<i>B. enteritidis</i> ; 3 agar cultures	4	"	Negative	Positive; almost pure in 1 tube.
142.....	<i>B. enteritidis</i> ; 3 agar cultures	5	"	"	Negative
142.....	<i>B. enteritidis</i> ; 3 agar cultures	6	"	"	"
142.....	<i>B. enteritidis</i> ; 3 agar cultures	7	"	"	Positive; almost pure in 2 tubes.
142.....	<i>B. enteritidis</i> ; 3 agar cultures	10	"	"	Negative
143.....	<i>B. paratyphosus</i> B; 3 agar cultures	4	Negative	Positive; 1 colony	Positive; pure in 1 tube; mixed in 1 tube.
143.....	<i>B. paratyphosus</i> B; 3 agar cultures	5	"	Positive; numerous colonies	Positive; mixed in 2 tubes.
143.....	<i>B. paratyphosus</i> B; 3 agar cultures	6	"	Negative	Negative
143.....	<i>B. paratyphosus</i> B; 3 agar cultures	7	"	"	Positive; almost pure in 2 tubes.
144.....	<i>B. paratyphosus</i> A; $\frac{1}{2}$ broth culture	1	"	.....	Positive; pure cultures.

Both *B. enteritidis* and *B. paratyphosus* B were isolated as late as the seventh day after feeding. In the first or second stool passed after infection by feeding (about 24 hours later) the number of colonies of the bacillus fed might equal on the Endo plates the total number of colonies of the colon type. After 48 hours, however, the bacilli fed had decreased to such a degree that none of their colonies appeared on the Endo plates and after four days they were evidently present in exceedingly small numbers. It is appar-

ent that the intestinal tract of the dog under normal conditions does not offer a favorable environment for bacilli of this type. *B. paratyphosus* A is evidently eliminated from the fecal flora of the dog more quickly than the B type or *B. enteritidis* as it has not been isolated later than 24 hours after feeding.

It is quite possible that this brilliant grèen enrichment medium may prove of value for investigations in regard to the presence of bacilli of the paratyphoid-enteritidis type in foods suspected of having caused poisoning or in water and ice subject to pollution. In material in which *B. lactis aërogenes* is present in great numbers, as in the poorer grades of milk, this medium should be used with caution because of the danger of overgrowth. In systematic investigations of this character this enrichment medium might be advantageously combined with the Loeffler malachite green agar plates.

#### SUMMARY.

1. As a selective dye for the isolation of bacilli of the paratyphoid-enteritidis type, brilliant green exhibits a greater degree of specificity than malachite green (Höchst 120).

2. Brilliant green glucose broth, prepared according to methods described in this paper, constitutes an enrichment medium of marked selective propensity for the paratyphoid-enteritidis group. A greater degree of success was attained in isolating *B. paratyphosus* B and *B. enteritidis* from the feces of dogs fed with these bacilli by the use of this enrichment medium than with Loeffler's malachite green-nutrose-lactose agar medium. Feces containing so few paratyphoid or enteritidis bacilli that none of their colonies appeared on the Endo plates or even on the Loeffler plate medium gave pure or nearly pure cultures with this enrichment medium.